Safety Report for PAGE GelRed®

DOMINIQUE DUTSCHER SAS

A summary of mutagenicity and environmental safety test results from Biotium and two independent laboratories for the nucleic acid gel stain PAGE GelRed®

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Biotium

Overview

Ethidium bromide (EB) has been the stain of choice for nucleic acid gel staining for decades. The dye is inexpensive, sufficiently sensitive and very stable. However, EB is also a known powerful mutagen. It poses a major health hazard to the user, and efforts in decontamination and waste disposal ultimately make the dye expensive to use. To overcome the toxicity problem of EB, scientists at Biotium have developed a series of safer nucleic acid gel stains, including GelRed® and GelGreen® for staining DNA in agarose gels, and PAGE GelRed® for staining DNA in polyacrylamide gels. This report describes the laboratory safety testing of PAGE GelRed®. Visit www.biotium.com to view the safety report for Biotium's original GelRed® and GelGreen® nucleic acid gel stains.

Dye Design Principle

Biotium scientists recognized that a fundamental approach for making a gel stain safe is to eliminate or minimize the chance for the dye to interact with genomic DNA in living cells. Based on this design principle, chemists at Biotium incorporated structural features to make the dyes impermeable to latex gloves, nitrile gloves, and cell membranes.

In the design of the original GelRed® and GelGreen® dyes, we achieved the dyes' membrane impermeability mainly by making the dyes physically large. While this strategy works extremely well to improve the dyes' safety and at the same time produces exceptional gel staining sensitivity for agarose gels, the relatively large size of GelRed® and GelGreen® make the dyes difficult to penetrate into the more densely packed polyacrylamide gels, rendering the dyes less optimal for PAGE gel staining. In designing the PAGE GelRed® dye, we used a novel approach to make the dye membrane impermeable without making the dye large. Importantly, the new design strategy still ensures that the PAGE dye possesses essential properties for gel staining, including good sensitivity, stability and compatibility with existing instruments and downstream sample analysis.

Safety Tests

PAGE GelRed® was subjected to a series of tests by Biotium and by two independent testing services to assess the dye's safety for routine handling and disposal. These tests include: 1) glove penetration test; 2) cell membrane permeability; 3) Ames test; and 4) environmental safety tests. Results of the tests are summarized in Table 1 below. The data show that PAGE GelRed® is non-membrane permeable, non-toxic and non-mutagenic, thus validating the dye design principle. The highest dye concentrations shown to be non-mutagenic in the Ames test for PAGE GelRed® dye are 15 times higher than the 1X working concentration used in gel staining. Detailed test results are described on the following pages.

Conclusion

PAGE GelRed® is a next generation nucleic acid gel stain designed for staining DNA in polyacrylamide gels. It possesses novel chemical features designed to minimize the chance for the dye to interact with nucleic acids in living cells. Test results confirm that the dye is unable to penetrate latex gloves, nitrile gloves, or cell membranes.

In the AMES test, PAGE GelRed® is noncytotoxic and nonmutagenic at concentrations well above the working concentrations used in gel staining. This is in contrast to SYBR® Safe, which has been reported to show mutagenicity in several strains in the presence of S9 mix (1). SYBR® Safe was reported to be non-mutagenic in Syrian hamster embryo (SHE) cells and L5178YTK +/- mouse lymphoma cells (2). However, in these assays mutagenicity was only tested for concentrations of SYBR® Safe below the 1X working concentration of 0.66 ug/mL. This is because excessive toxicity was observed at concentrations above 0.333 ug/mL in SHE cells and above 0.25 ug/mL in L5178YTK +/- mouse lymphoma cells (1,3).

Furthermore, PAGE GelRed® has successfully passed environmental safety tests in compliance with CCR Title 22 Hazardous Waste Characterization. As a result, the dye is not classified as hazardous waste, and thus can be safely disposed of down the drain or as regular trash, providing convenience and reducing cost in waste disposal.

References

1. Report: SYBR Safe DNA Gel Stain, Assessment of Mutagenicity and Environmental Toxicity. http://probes.invitrogen.com/media/publications/494.pdf
2. Beaudet, MP, Hendrickson, JE, Ruth, JL. Safety testing of SYBR Safe, a non-hazardous alternative to ethidium bromide. http://probes.invitrogen.com/media/publications/519.pdf
3. The working concentration of SYBR Safe was calculated using the absorbance of the 1X solution, the extinction coefficient for SYBR dyes (70,000) and the molecular weight of SYBR Safe reported in reference 4 (MW 505).
4. Evenson, WE, Boden, LM, Muzikar, KA, and O'Leary, DJ. 1H and 13C NMR Assignments for the Cyanine Dyes SYBR Safe and Thiazole Orange. The Journal of Organic Chemistry 2012 77: 10967-10971.

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Table 1. Summary of PAGE GelRed® Safety Test Results

	Latex/ Nitrile Glove Penetration	Cell Membrane Permeability	Cytotoxicity	Ames Test	Hazardous Waste Screening (aquatic toxicity test)
PAGE GelRed	Impermeable	Impermeable	Nontoxic	Nonmutagenic	Nontoxic to aquatic life

This document is intended to provide a brief summary of the safety data on PAGE GelRed® dye obtained from several independent laboratories. If you wish to see the original test reports, you may contact Biotium technical support at techsupport@biotium.com.

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SYBR is a registered trademark of Thermo Fisher Scientific.

Glove Penetration Test

Purpose

Latex and nitrile gloves are commonly worn by researchers in laboratories as protective gear. Thus, it is important to show PAGE GelRed® does not readily diffuse through these glove materials.

Method

A finger of a latex or nitrile glove containing TAE buffer was dialyzed against TAE buffer containing 5X PAGE GelRed® for 48 hours. The solution in the finger was then analyzed for presence of the dye by fluorescence. As a reference, the fluorescence of the dye at 5X was also measured. To increase the sensitivity of dye detection, all fluorescence measurements were made in the presence of 100 ug/mL dsDNA from calf thymus.

Results

The results of the test show that PAGE GelRed® is unable to penetrate latex gloves (Figure 1).

Conclusion

Latex and nitrile gloves provide an effective barrier to PAGE GelRed®.

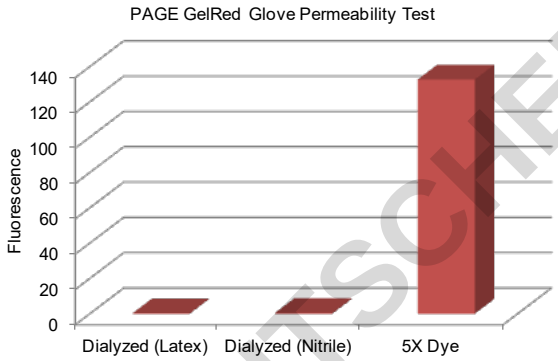


Figure 1. Relative fluorescence of solutions dialyzed in latex or nitrile glove fingers against 5X PAGE GelRed® and the relative fluorescence of the corresponding 5X dye solution as a reference. The data show that the amount of the fluorescence for the dialyzed solutions is negligible compared to the fluorescence of the 5X dye solution, suggesting that PAGE GelRed® does not efficiently penetrate latex or nitrile gloves at 5X concentration.

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Cell Permeability Test

Purpose

The purpose of this test is to determine whether PAGE GelRed® can cross cell membranes to stain nuclear DNA.

Method

Hela cells were incubated at 37°C with PAGE GelRed® or SYBR® Safe. The dye concentrations were 1X based on the respective dye concentrations used for gel staining for each dye. SYBR® Safe was used as a control because we have previously shown that it stains DNA in live cells. Cell staining was followed by fluorescence microscopy. SYBR® Safe was imaged using a FITC filter set. PAGE GelRed® was imaged using a Cy3 filter set. Cells were observed under phase contrast to ensure similar cell densities in each imaging field (not shown).

Results

Microscopic images obtained following 5 minutes and 30 minutes of incubation are shown in Figure 2. Bright green fluorescent staining was observed in nuclei and cytoplasm of cells throughout the culture well for SYBR® Safe after five minutes of incubation. Conversely, PAGE GelRed® did not stain living cells even following 30 minutes of incubation. Staining of rounded-up dead cells present sporadically in the cultures was observed for PAGE GelRed®, suggesting that the dye can only enter cells that have compromised plasma membrane integrity, which is observed for other non-membrane-permeable nucleic acid dyes. Examination of cell morphology by phase contrast microscopy showed no increase in cell death after overnight incubation with 1X PAGE GelRed® compared to untreated cells.

Conclusion

PAGE GelRed® is unable to penetrate cell membranes.

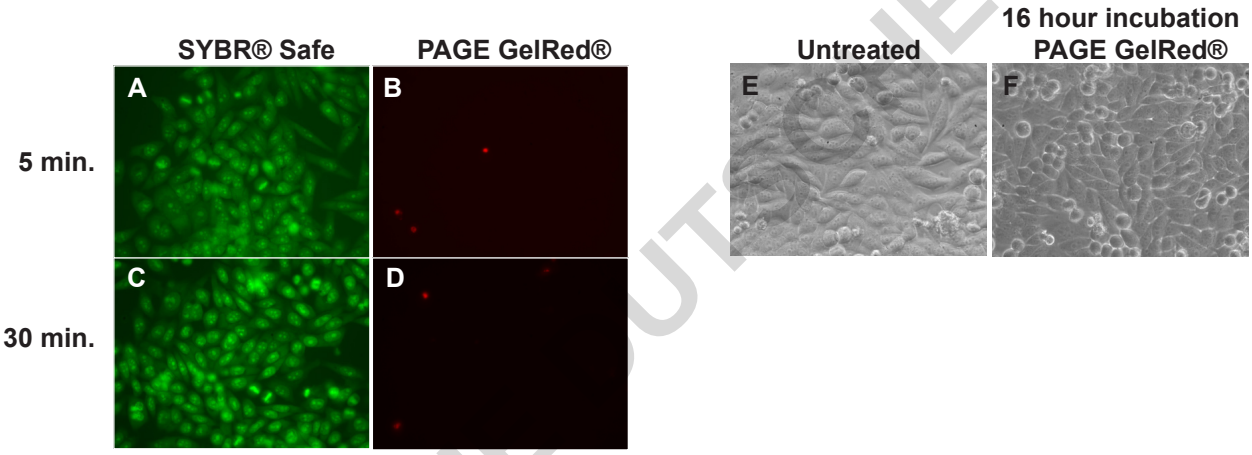


Figure 2. HeLa cells were incubated at 37°C with 1X SYBR® Safe or 1X PAGE GelRed®. Cells were imaged following incubation for 5 minutes (A-B) and 30 minutes (C-D). SYBR® Safe rapidly penetrated cell membranes as evident from the bright green staining of nuclei and cytoplasm after five minutes of incubation. However, PAGE GelRed® was unable to cross cell membranes, as shown by the absence of fluorescence staining in healthy cells. Staining was observed in dead cells present sporadically in the cultures, as is observed with other non-membrane permeable nucleic acid dyes. The presence of cells in the field of view was confirmed by phase contrast microscopy. The exposure time for images captured after 5 minutes was 800 msec. The exposure time for images captured after 30 minutes was 200 msec for SYBR® Safe and 400 msec for PAGE GelRed®. No increase in cell death was observed after overnight incubation with 1X PAGE GelRed® compared to untreated cells (E-F).

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Ames Test

Purpose

The Ames test is a standard assay to assess the mutagenic potential of chemicals. As cancer is often associated with DNA damage, the test can be used to estimate the carcinogenic potential of a chemical compound.

Test System

Testing was performed by BioReliance Corporation (Rockville, MD). The test employed *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537, and *Escherichia coli* strain WP2 uvr A. These strains carry mutations in the operon coding for histidine biosynthesis. When these bacteria are exposed to mutagenic agents, under certain conditions reverse mutation from amino acid (histidine) auxotrophy to prototrophy occurs, giving colonies of revertants. Tester strains TA98 and TA1537 are reverted from auxotrophy to prototrophy by mutagens that cause frameshift mutations. Tester strain 1535 and *E. coli* strain WP2 uvr A are reverted by mutagens that cause basepair substitutions. Tester strain TA100 is reverted by mutagens that cause both frameshift mutations and basepair substitutions. In order to test the mutagenic toxicity of metabolized products, Aroclor-induced rat liver S9 fraction, a rat liver extract, was used in the assays. The S9 fraction contains a mixture of several enzymes and is known to be able to convert some chemicals into mutagens.

Test Articles and Vehicle

PAGE GelRed® in water was used at the following concentrations: 0 (control), 0.1, 0.3, 0.65, 1.3, 2.5, 5, 7.5, and 10 uM per plate. The working concentration of PAGE GelRed® used for gel staining is 0.65 uM.

Test Procedure

Test article dilutions were prepared immediately before use and delivered to the test system at room temperature under yellow light. S9 or Sham mix (0.5 mL), tester strain (100 uL) and vehicle or test article dilution (50 uL) were added to 2 mL of molten selective top agar at 45+/-2°C. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. When plating the positive controls, the test article aliquot was replaced by 50 uL of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for approximately 48 to 72 hours at 37+/-2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted.

The condition of the bacterial background lawn was evaluated for evidence of test article toxicity by using a dissecting microscope. Precipitate was evaluated by visual examination without magnification. Revertant colonies for a given tester strain and activation condition were counted either entirely by automated colony counter or entirely by hand unless the plate exhibited toxicity. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually.

Evaluation of Results

For a test article to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test article. Data sets for tester strains TA1535 and TA1537 were judged positive if the increase in main revertants at the peak of the dose response is equal to or greater than 3.0-times the mean vehicle control value. Data sets for tester strains TA98, TA100, and WP2 uvr A were judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 2.0-times the mean vehicle control value.

Criteria for a Valid Test

The following criteria must be met for the mutagenicity assay to be considered valid. All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (rfa) and the deletion in the uvr B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 uvr A cultures must demonstrate the deletion in the uvr A gene. All cultures must demonstrate the characteristic mean number of spontaneous revertants in the vehicle controls as follows (inclusive): TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21; WP2 uvr A, 10-60. To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/mL. The mean of each positive control must exhibit at least a 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control. A minimum of three non-toxic dose levels is required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50% reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) At least a moderate reduction in the background lawn (background lawn code 3, 4, or 5).

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Results from Ames Test for PAGE GelRed®

See page 5 for a description of the test system. No precipitate or toxicity was observed at any dose of PAGE GelRed® either with or without S9 activation. PAGE GelRed® was non-mutagenic at all doses tested in all strains tested, with or without S9 activation. The average number of revertants per plate is shown below.

Table 2. Average revertants per plate +/- standard deviation for PAGE GelRed® without S9 activation

Dose (uM/ plate)	TA98	TA100	TA1535	TA1537	WP2 uvr A
Vehicle Control	26 +/- 6	92 +/- 6	8 +/- 4	16 +/- 2	27 +/- 10
0.1	35 +/- 3	89 +/- 7	10 +/- 1	14 +/- 3	35 +/- 4
0.3	34 +/- 10	88 +/- 19	8 +/- 4	17 +/- 8	26 +/- 1
0.65*	32 +/- 6	100 +/- 11	10 +/- 6	18 +/- 8	30 +/- 4
1.3	31 +/- 7	78 +/- 2	15 +/- 1	14 +/- 3	33 +/- 14
2.5	38 +/- 2	91 +/- 1	8 +/- 1	14 +/- 4	35 +/- 10
5.0	29 +/- 4	88 +/- 9	9 +/- 4	15 +/- 0	23 +/- 13
7.5	35 +/- 2	92 +/- 10	12 +/- 4	22 +/- 11	41 +/- 4
10	36 +/- 2	82 +/- 11	12 +/- 0	19 +/- 6	29 +/- 1
Positive Control	116 +/- 5	476 +/- 4	212 +/- 193	334 +/- 61	419 +/- 35

* 1X Concentration for Gel Staining

Table 3. Average revertants per plate +/- standard deviation for PAGE GelRed® with rat liver S9 activation

Dose (uM/ plate)	TA98	TA100	TA1535	TA1537	WP2 uvr A
Vehicle Control	46 +/- 17	104 +/- 1	13 +/- 8	13 +/- 4	30 +/- 6
0.1	33 +/- 4	95 +/- 7	12 +/- 4	19 +/- 1	43 +/- 8
0.3	42 +/- 12	97 +/- 9	12 +/- 4	17 +/- 0	31 +/- 4
0.65*	39 +/- 4	97 +/- 14	9 +/- 2	13 +/- 1	32 +/- 6
1.3	46 +/- 6	102 +/- 4	14 +/- 2	17 +/- 1	29 +/- 1
2.5	41 +/- 5	90 +/- 13	12 +/- 4	21 +/- 1	48 +/- 6
5.0	38 +/- 0	88 +/- 26	16 +/- 6	12 +/- 0	38 +/- 7
7.5	38 +/- 1	109 +/- 3	10 +/- 6	11 +/- 5	44 +/- 1
10	40 +/- 11	99 +/- 4	11 +/- 6	15 +/- 4	32 +/- 0
Positive Control	410 +/- 23	411 +/- 13	60 +/- 4	75 +/- 13	139 +/- 30

* 1X Concentration for Gel Staining

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Aquatic Toxicity Test

Purpose

This test assesses the acute toxicity of PAGE GelRed® to aquatic life. The results of the test are used to determine if the dye can be directly released into the environment for disposal under California Code of Regulations (CCR) Title 22.

Test Specifications

Testing was performed by Nautilus Environmental (San Diego, CA).
Number of replicates and fish: 2 replicates with 10 fish each (20 fish total per concentration)
Method used: CA Dept. of Fish & Game, 1988 Acute Procedures;
Regulatory guidelines: CCR Title 22 Hazardous Waste Characterization
PAGE GelRed test specifications:
Test start date, time: 7/30/13, 12:10
Test end date, time: 8/3/13, 12:00
Test organism: *Pimephales promelas* (Fathead minnow)
Organism mean length/weight: 29.6 mm/0.333 g
Test concentration: 750, 500, and 250 mg/L PAGE GelRed 1X staining solution (i.e., 750, 500, and 250 uL/L of 1X staining solution); plus Lab Control

Passing requirements

Sample must result in greater than 50% survival at a concentration of 500 mg/L (LC_{50} > 500 mg/L) to be “not hazardous” to aquatic life under CCR Title 22 regulations.

Results

The results are summarized in Table 4 below. PAGE GelRed® gave LC_{50} > 750 mg/L and therefore passed the Title 22 test.

Conclusion

PAGE GelRed® 1X staining solution is classified as nonhazardous to aquatic life and can be safely released into the environment under CCR Title 22 regulations.

Table 4. Summary of aquatic toxicity test results

Sample	Concentration (mg/mL)	% Survival
Lab control	N/A	100
PAGE GelRed®	250	100
	500	100
	750	100

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